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(57) Abstract

A method is provided for measuring an analyte in sample comprising adding substantially transparent particles to a sample in solution or suspension, said particles having an affinity for said analyte; fractionating the particles from the solution or suspension to form a particle-rich fraction and a substantially particle-free fraction; optically reading the particle-rich fraction at a first wavelength; optically reading the substantially particle-free fraction at a second wavelength; and correlating the readings through the particle-rich fraction and the substantially particle-free fraction to obtain a quantitative determination of the analyte originally present in the sample.

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TWO-PHASE OPTICAL ASSAY METHOD AND APPARATUS

This is a continuation-in-part of application Serial No. 08/073,450, filed June 8, 1993, the entire contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method and apparatus for quantifying analytes in a sample. It is particularly useful for measuring glycosylated and unglycosylated hemoglobin in a blood sample.

Description of the Background Art

A. Assay Methods Generally

Many analytes of interest have one or more characteristic wavelengths of absorbance or fluorescence. In theory, the amount of such an analyte in the sample may be determined by performing a suitable optical measurement on the sample. In practice, the accuracy of this simple analysis is impaired if any of the other components of the sample also absorb or fluoresce at that wavelength.

Therefore, it is customary to use a solid phase affinity reagent to separate the analyte from interfering substances.

In a conventional solid-liquid mini-chromatography assay, an affinity reagent is attached to a solid phase (e.g., beads in a column), and the sample is introduced into a liquid phase which is passed over the solid phase. Components which are strongly bound by the affinity reagent will be seriously retarded in the passage, weakly bound constituents will move more quickly through the liquid phase, and components which do not interact at all with the affinity reagent will move fastest of all. The constituents of the sample can therefore be separated, with the least bound constituent collected in the earlier effluent fractions and the strongly bound constituents appearing later. The presence of an analyte of interest in the sample is then detected by placing a detector in the flow path between the column and the effluent collector, or by collecting

the fractions in separate vessels, and measuring the amount of analyte in each vessel.

Of course, a given analyte will not flow out all at once. There will be trace amounts of analyte in one fraction, a more substantial amount in another fraction, the analyte will peak in a third fraction, and then will taper off in later fractions. If the constituent is to be quantified, then it is necessary to measure the amount (e.g., by absorbance) of analyte in each fraction as it exits the column, and then integrate the values over time.

In general, while the presence of analyte in a retained fraction on a minicolumn may be visible, it is not possible to measure the analyte while it is thus still bound to derivatized particles in the minicolumn.

In the example given above, the process of analyzing the sample is continuous. However, batch processes are also possible. In this case, there is no need for the liquid phase to flow at all. Rather, the sample is introduced into the liquid phase, which is contacted with the solid phase. After a suitable incubation period, the two phases are separated (e.g., by decanting the liquid and washing off the solid). An eluting agent is then added to the solid phase, and the amount of analyte eluted is measured.

In another variation, no eluting agent is added after removal of the liquid phase. Rather, the analyte is labeled in situ with a second, labelled affinity reagent, and the solid phase is washed to removed excess labelled reagent. The amount of label still associated with the solid phase is then measured. Usually the label is either a radioisotope, and is read with a geiger counter, or is an enzyme, which converts a substrate reagent to a colored reaction product which is read colorimetrically. This is the classic heterogeneous "sandwich" assay.

All of the solid phase assays described above have the disadvantage of requiring considerable handling of the sample. Such handling can be time-consuming and expensive, and can increase the risk of contamination of the sample.

A number of assays have been proposed where the solid phase bound analyte is measured without separation of the solid phase.

Such assays require that a detectable signal be generated by the binding of the analyte to the affinity reagent, but not by unbound analyte.

Frank et al., in U.S. Patent No. 4,283,382, disclose a fluorescent immunoassay using latex beads which are labelled with the fluorophore and also coupled or coated with an analyte binding reagent such as an antigen or antibody. If the sample contains the corresponding polyvalent analyte, the latex beads are agglutinated, and the agglutinate settles to the bottom. (Thus, agglutination is the signal mentioned above.) The fluorescence of the supernatant is then compared with that of the settled bead volume. However, the assay is not quantitative, agglutination is relied upon, the beads are not transparent and are so small that light loss from multiple reflections is likely, and only one wavelength of fluorescent light is measured.

Anderson, in U.S Patent No. 3,862,303, discloses a binding assay in which the analyte being measured binds to latex beads which have a specific affinity binding agent attached. The binding analyte is detected by its alteration of the effective density of the bead. After incubation with sample, the beads are centrifuged in a liquid having a density gradient. The beads gravitate to the appropriate layer, depending on the amount of analyte bound. The binding may be observed without separating bound from free analyte. This method places stringent requirements on the size and density of the beads, and it is the opacity of the beads, not the specific absorbance of the analyte, that provides the assay results. Making the beads transparent would defeat the assay. The beads are distributed throughout the density gradient column.

Giaever, in U.S. Patent No. 4,115,535, discloses the use of two different types of particles in a qualitative binding assay. Both particles bear an analyte-binding agent such as an antibody. The first type facilitates separation, and may be magnetic or high density particles. The second type provides detectability, such as fluorescent or colored particles. Theoretically the analyte causes agglutination of both types of particles, so that the first type will draw the second type

along. While the inventor alludes to a density-based separation, the separations disclosed are generally electromagnetic.

B. Glycohemoglobin Assays

Glycated or glycosylated hemoglobin is of particular clinical interest in managing patients affected with diabetes mellitus. Measuring glycosylated hemoglobin is a clinically useful means of assessing glycemic control in diabetic patients over a period of time. Glycosylated hemoglobin values reflect blood glucose levels over the circulatory half-life of the erythrocyte (about 60 days) and correlate significantly with mean blood glucose levels during that time. Therefore, measurement of glycosylated hemoglobin provides a means, independent of multiple measurements such as patient records of self-monitored blood glucose, for assessing the overall efficacy of therapy. Factors such as diet, exercise, insulin regimen and stress can affect glycemic control, and therefore the glycosylated hemoglobin values.

In uncontrolled or poorly-controlled diabetics, glycosylated hemoglobin values may be two or three times as high as in non- diabetics, while meticulously controlled diabetics may have glycosylated hemoglobin values near or in the normal range. Uncontrolled or poorly-controlled diabetics brought under better control will exhibit a gradual drop in glycosylated hemoglobin values, reaching a new equilibrium in approximately eight weeks. There is significant evidence that maintaining good glycemic control has a positive impact on the development of the long-term complications of diabetes.

There have been several previous attempts to measure glycosylated hemoglobin along with unglycosylated hemoglobin. Deutsche, in U.S. Patent 4, 762,798, discloses a method for measuring hemoglobin and glycohemoglobin almost simultaneously by first measuring total optical absorbance followed by adding an adsorbent for unglycosylated hemoglobin. The mixture is mixed and decanted to leave only the glycohemoglobin, which is then measured by optical absorbance. In this process, the hemoglobins must be separated from each other for measurement,

and time must elapse between the measurements to remove hemoglobin. This assay thus requires time between measurements to remove unglycated hemoglobin, making the time required for each assay unduly long.

Sutherland et al., in U.S. Patents 4,775,637 and 4,818,710, disclose a system for measuring glycosylated hemoglobin in a solution containing non-glycosylated hemoglobin. In this system, a cuvette has antibodies to glycosylated hemoglobin attached to one side of the cuvette. The optical properties of the specifically bound glycoslyated hemoglobin on the antibody-coated side are compared to those of the hemoglobin on the uncoated side. Non-glycosylated hemoglobin non-specifically binds to both sides of the cuvette. The wavelength of light is selected so that the hemoglobin bound to the cuvette would absorb the light. This process measures without chromatography or other liquid separation; there is no settling or centrifugation. The cuvette presents only a relatively small surface area for the binding of the analyte.

Curtiss et al., in U.S. Patent No. 4,778,752, disclose monoclonal antibodies specific for glucitollysine; this epitope is found on glucohemoglobin but not on hemoglobin A_0. The antibody is used in a radioimmunoassay (RIA). The description of the RIA refers to "washing", so that there is a physical separation of bound and free analyte.

Wagner, in U.S. Patent No. 4,861,728, discloses an assay for glycosylated hemoglobin and non-glycosylated hemoglobin simultaneously in the same vessel. One reagent is a solid phase which binds both HbA_0 and HbA1c. The solid phase may be a glass or a polymer, and, while a dipstick shape is preferred, the solid phase may be of any suitable shape. The reagent is reacted with the sample, and the bound hemoglobins are separated from the mixture. The color of the solid phase is then read. The bound hemoglobins are incubated with a second reagent which reacts only with the glycoside portion of HbA1c, and is conjugated with a fluorescent dye. The dye absorption of incident light indicates the level of HbA1c. While HbA_0 is not physically separated from HbA1c, the Hbt is separated from the rest of the sample.

Wagner et al., in U.S. Patent No. 4,806,468, disclose measuring hemoglobin by adsorption and glycosylated hemoglobin in the same container by adsorption at different wavelengths. This assay depends on the finding that the peroxidase activity of hemoglobin is blocked by anti-Hb, while the peroxidase activity of HbA1c is retained when it binds to anti-HbA1c. Moreover, anti-HbA1c inhibits the binding of anti-Hb to HbA1c. In the assay, the red blood cells are lysed to release the Hb, and a mixture of anti-Hb and anti-HbA1c is added. The total Hb (A_0 and A1c) is measured by determining the absorbance at 416 nm or 540 nm. A peroxidase substrate is added, and the absorbance of the peroxidase reaction product is detected at a different wavelength, e.g., 620 nm. While two different wavelengths are used, there is no sequestration of glycohemoglobin in one portion of the vessel, or separate imaging of different zones of the vessel. The antibodies are provided in solution form rather than in the solid phase.

A number of workers have used a chromatographic column to separate glycosylated hemoglobin from non-glycosylated hemoglobin by means of an affinity reagent specific to the glycosylated form bound to the solid phase: Dean et al., U.S. Patents Nos. 4,269,605 and 4,629,692; Klen et al., Clinical Chemistry, 28(1): 2088-2094 (1982); Kricka et al., Clinical Chemistry 37(9): 1991 (1991); Pecoraro et al, Diabetes, 28: 1120 (1979); Fluckiger et al., Diabetes 33: 73-76 (1984) and Saunders, Clinical Chemistry 37: 1531 (1991).

Lewis et al. in U.S. Patent No. 4,847,209, disclose an agglutination assay for glycosylated hemoglobin in the presence of hemoglobin by binding a monoclonal antibody to a latex particle. This antibody specifically binds glycosylated hemoglobin and allows an optical measurement of glycosylated hemoglobin in the presence of hemoglobin. However, a number of calculations are required to obtain % HbA1c, and a latex blank must be included in each run.

Deeg et al., in Clinical Chemistry 30(5): 790-793 (1984) disclose the measurement of glycated hemoglobin by adding a reactant which blocks normal hemoglobin from binding to haptoglobin but not glycated hemoglobin.

Sanders, in U.S. Patent No. 4,407,961, separates glycoslyated hemoglobin from non-glycosylated hemoglobin using a column which adsorbs the non-glycosylated form. The absorbance of the eluate and of whole blood lysate are compared.

C. Two-Wavelength Measurements

There have been a few instances of measuring at more than one wavelength to correct for unknown components in the optical path.

Kamensky (1965) observed human cells passing through a flow cytometer by measuring their absorption at 2537 Å and their scattering at 4100 Å. The former parameter was used to estimate nucleic acid content, and the latter to derive the cell size or mass.

Patau (1952) discusses the problems associated with absorption microphotometry of irregularly shaped objects. One error is caused by the non-uniformity of the dye distribution in the object. Patau suggests correcting for this error by measuring absorption at two different wavelengths, with one preferably being twice the other. The measurements must be made both with and without the object. Another error is caused by the stray light entering the photo-receiver without passing through the object. Patau emphasizes that the larger, less transparent the object the larger the errors. Ornstein (1951) discusses the mathematics of the distributional error, and provided inspiration to Patau.

SUMMARY OF THE INVENTION

According to the present invention, an affinity reagent which specifically binds the analyte is immobilized on substantially transparent particles, such as beads, to form a solid phase particulate affinity reagent. This solid phase reagent is then incubated with sample sufficiently long for the analyte to react with the reagent. The mixture is then fractionated (e.g. by settling or centrifugation), so that the particles collect within one zone, the "particle-rich" zone, of the reaction vessel, leaving another zone, the "particle-free" zone, substantially free of particles. It should be noted that

the present invention does not require that the binding of the particle-bound affinity reagent to the analyte result in agglutinate formation; substantially all particles, whether bearing bound analyte or not, are transported to the particle-rich zone. Also, fractionation does not require physical removal of one phase from the other.

As a result of the binding activity of the affinity reagent on the particles, the analyte is concentrated in the particle-rich zone, whereas unbound constituents of the sample are in substantially equal concentrations in the particle-rich and particle-free zones. Also, unbound analyte is found in substantially equal concentrations in the particle-rich and particle-free zones. The analyte, or the complex of the analyte and the affinity reagent, is assumed to have a characteristic wavelength of absorption, transmission, fluorescence or phosphorescence. The particle-rich fraction is "read" at this wavelength; the optical path passes through one or more of these particles.

As long as the potentially interfering substances in the sample are not significantly bound by the analyte, they will be present in substantially equal concentrations in both zones. Therefore, by measuring the <u>difference</u> in absorbance (or whatever) between the two zones, the interference is eliminated.

The use of a particulate reagent results in a greatly increased surface area for the capture of the analyte, as compared to, say, a microtiter plate. Preferably, the particles are porous, to further enhance analyte capture.

A potential problem with the method as described above is that there will be a loss of light received from the particle-rich fraction as a result of the absorbance and scattering of light by the particles. Of course, the more transparent the particle, and the shorter the nominal optical path through the particle-rich zone, the less particle absorbance will be a concern. However, the path of incident light can be significantly perturbed by a suspension of small spheres, thanks to reflection and refraction. If more and smaller particles are used, to increase surface area, light scattering will worsen.

One solution to this problem is the use of a "blank" which contains the affinity reagent-labeled particles, but not the analyte. However, this is inconvenient. A better solution is to read the absorbance (or fluorescence) of the particle-rich fraction at a second ("control") wavelength which is insensitive to the concentration of the analyte.

It is also possible, for further precision of analysis, to optically examine the particle-free fraction at one or more control wavelengths as well.

It is further within the contemplation of the invention to spread the particle-rich fraction into a relatively optically thin layer, so as to reduce multiple reflections.

In a preferred embodiment, the sensitivity of the assay is increased by decreasing the volume of the phase to which the constituent present in lesser amount would migrate, thus concentrating this constituent and increasing the sensitivity of its optical measurement.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the combination of a reaction chamber with a long, thin tube in a single assembly.

Figure 2 shows an assay apparatus in the form of a long, thin tube using an LED as the light source and a photodiode for measurement.

Figure 3 shows a comparison of results obtained in Example 1 of the present invention as compared to a standard commercial method for testing for glycohemoglobin.

Figure 4 shows a comparison of results obtained in Example 2 of the present invention as compared to a standard glycohemoglobin procedure.

Figure 5 shows the results obtained in Example 3 as compared to a standard procedure for measuring glycohemoglobin.

Figure 6 shows the correlation between hemoglobin with a standard spectrophotometric measurement and the fluorescence measurement as obtained in Example 4.

Figure 7 shows measurement results of Example 5 as compared with a standard commercially available glycohemoglobin kit.

Figure 8 shows the results obtained in Example 6 using the same comparison as in Figure 7.

Figure 9 shows the results obtained in Example 7 as compared with the Pierce Glycogel method.

FIGURE ELEMENTS

2. reaction/measuring apparatus
4. reaction chamber
6. cap
8. loop attaching cap to reaction chamber
10 plug separating insides of two chambers
12. sample
14. bore
18. mixing means
20. bubbles
22. bottom of reaction suspension
24. string attached to plug
26. frit in measuring chamber
28 particles
30. liquid
32. bottom of measuring chamber
34. closure for bottom of apparatus
36. optical path for particle-rich fraction
38. optical path for particle-free fraction
50. light emitting diode
51. light emitting diode
60. parallel optical trains
62. alternate optical path

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides a method for improving the sensitivity of conventional chromatographic analysis techniques by converting the analyte liquid into two phases by the addition of particles to which an affinity reagent is attached. The particles are substantially transparent to the incident radiation used in the assay, so that there is no need to form agglutinates. The fluid is then converted to a particle-rich

zone and a particle-free zone; the analyte is concentrated by the action of the affinity reagent into the particle-rich zone, whereas unbound constituents in the sample are dispersed relatively equally between the particle-rich and the particle-free zones. Where more than one analyte is to be determined, particles bearing affinity reagents for each analyte are added to the sample.

The present invention differs from conventional assays in (1) measuring at two different wavelengths and (2) using particles which are substantially transparent to the radiation wavelength used for measuring. By using particles which are substantially transparent to the light used for measurement, there is no requirement for physically removing the unbound fraction, or for collecting the various fractions in different containers. This, of course, could not be accomplished with particles which are not transparent to the wavelength of radiation used for the assay. As noted above, the transparency of the particles used to the incident radiation used for the assay much be such that the differences in transmission or excitation must be readily measurable at the desired wavelength.

In prior methods using dual wavelengths, the purpose of using dual wavelengths was to overcome inaccuracy created by the accidental presence of spurious particles (e.g., turbidity) or the random presence of molecular species with overlapping absorption spectra. In the present invention, the non-absorbing wavelength acts as an estimate of 100% optical transmission, i.e., 0 optical density, and is a situation in which the dimensions and optical path of the liquid are otherwise not precisely known. This takes the place of a measurement of the same optical path (through the same gel particles) in the absence of absorbing species. It is quite evident that the two color approach is more rapid and convenient than conventional measuring techniques, and is feasible without changing the optical path.

Among the advantages of the present invention are that it uses much less of the affinity reagent-particle reagent than do certain other methods, that it requires fewer manipulation steps and volumetric measurements, and that through concentration of

the analyte into a relatively small volume, it improves sensitivity. Thus, the method can be conducted by relatively unskilled personnel. The purpose of reading at a second wavelength different from that emitted by a fluorescent reagent is to reduce errors which may be introduced by differential light absorption of the different concentrations of sample in the two phases, or from the particles used.

When the sample is in suspension, the optical reading is conducted through both the particle-rich and the particle-free zones, and there need not be agglutination of the particles, but merely an affinity of the analyte for the reagent on the particles. By optically reading the sample at two different wavelengths as in a preferred embodiment, extraneous components in the sample, such as cellular elements in a blood sample, can be corrected for.

There are, of course, a number of conditions which could interfere with the assay as outlined above. For example, the constituents which were not bound at all by the affinity reagent would nevertheless be present in the particle-rich zone and, to the extent that they absorbed at the wavelength of interest, would cause the amount of the analyte of interest to be overestimated. Light would be lost by scattering off the surface of the particles, and through absorption in the interior of the particles. This too would cause the analyte reading to be exaggerated, if no correction were made. To complicate matters further, the amount of scattering could be a function of the amount of analyte captured by the particles through the affinity reagent.

Applicants have solved these problems by devising appropriate controls. For example, the contribution of extraneous constituents to absorption may be accounted for by measuring the absorbance in the particle-free zone, multiplying it by the free volume proportion of the particle-rich zone, and subtracting the resulting product from the absorbance of the particle-rich zone. Free volume proportion is determined experimentally by incubating the particles with analyte-free medium and measuring absorbance in both zones. This accounts for absorption of liquid in the interior of porous particles.

Another approach is to measure the absorption by the particle-rich zone of light at a different wavelength, which is not absorbed by the analyte, but is scattered to the same degree.

For assaying more than one analyte simultaneously, light absorption by the particle-free zone is measured. For each analyte, the optical contrast to be measured is matched to the compounds to be measured. The particle-free zone is measured and compared to the measurements taken for the particle-rich zone. Since each analyte attached to the particles absorbs or diffracts or deflects light at varying wavelengths, these wavelengths are used for detection and correlated with the concentration of the individual analyte in the sample. There is no need to elute the liquid from the particles, or to separate the analytes from each other by affinity to different particles in the system.

The present invention does not require separation of the particles from the sample fluid; the optical assay can be conducted in situ using different wavelengths of radiation for each individual analyte. No separation of individual analytes or particles is required.

Samples

The samples of the present invention may be a biological fluid, such as blood, urine, sweat, tears, milk, amniotic fluid or cerebrospinal fluid, or a nonbiological fluid such as a beverage, groundwater, or a solubilized soil sample.

Analyte

The analyte may be any analyte which can successfully be separated by a chromatographic separation process. Among these analytes are proteins such as hemoglobin, glycosylated hemoglobin, albumin, immunoglobulins and enzymes; lipids, such as triglycerides and lipoproteins, including high-density and low-density lipoproteins; therapeutic drugs, such as diphenylhydantoin, phenobarbital, tobramycin, lidocaine, procainamide, and the like; natural or synthetic steroids such as cortisol, aldosterone, testosterone, progesterone, estriol, etc.;

hormones, such as thyroid hormones, peptide hormones, insulin; antigens, antibodies, and other species which react naturally with a receptor.

Affinity Reagent

The affinity reagent may be any molecule which has a greater affinity for the analyte than for one or more of the other constituents of the sample. The principal classes of affinity reagents include lectins (where the analyte contains carbohydrate), antibodies (where the analyte is antigenic), antigens (where the analyte is an antibody), enzymes, co-enzymes, enzyme substrates and a member of five groups consisting of enzyme inhibitors (where the analyte is another, interactive member of that group, and nucleic acids (where the analyte is a complementary nucleic acid). Charged or hydrophobic substances have also been shown to be useful as affinity reagents.

Table I provides some combinations of reagents and constituents that can be used.

TABLE 1

Affinity on particle	Linker	Analyte(s)	Method of Measurement
Boronyl group			glycated vs non glycated
		a) hemoglobin	Absorption 570 nm Fluorescence quench
		b) globin	Refractive light loss
		c) albumin	Refractive light loss
		riboflavin	Specific fluorescense on particulate
Specific Antibody		Hemoglobin A1c	Absorption 570 nm
		Non Light Absorbing Antigen	Refractive light loss
Carbohydrate with free OH on adjacent cabon	Homodimer of boronyl	a) glycohemo-globin b) riboflavin	
Carbohydrate with free OH on adjacent carbon	Heterodimer a) boronyl b) lectin	Blood group substances	
Protein A	Specific Antibody	Transferrin	

Particles

The particles used in the present invention must be compatible with both the affinity reagent and the sample, and it must be possible to immobilize the affinity reagent onto the particle. The particle must be sufficiently transparent to permit the imaging of analyte, or of analyte-affinity reagent complex, borne by the particle. The particles used are transparent in some media. However, when the particles are immersed in the reaction medium, there is always some surface refraction because of a mismatch in the index of refraction between the solution and the particles. This phenomenon is demonstrated by the Sephadexes, including Sephadex G10, 25, 50, 75, 100 and 200, which have a swollen solid mass of approximately 25, 20, 9 6, 5 and 2.5% solids, respectively. Transmission of 560 nm light, in the same order for these particles in capillaries, is 7.2, 32.4, 78.3, 73.1, 84.4 and 89.9%. Transmission at 630 nm, again in the same order, is 8.1, 33.9, 77.7, 74.2, 86.6 and 91.7%. The transmission of Sephadex G75, in which the transmission seem anomalous when compared with the other, is composed of smaller spheres than the other examples. When it may be done without interfering with the assay, a refractive index-adjusting agent may be added to the medium.

For use in the present invention, the particles should be substantially transparent to the wavelength of radiation used for detection. The amount of the analyte of interest in the particle-rich zone may then be determined optically by correlating the absorbance of the particle-rich zone, at a wavelength characteristic of the analyte, with a suitable standard. In contrast to several of the procedures mentioned previously, there is no need to physically remove the unbound fraction, or to collect the various fractions in different containers. Of course, with the opaque particles typically used in the art, absorbance measurements are not practical.

It should be noted at this point that even if the particles used in the assays of the art were transparent to light, they would not necessarily be suitable for absorbance

studies. If the particles were very small, and were densely packed, there would be a great deal of light lost as a result of multiple reflections. Also, if the particles were placed in a column with too great a diameter, light passing through the particles would be diffused as a result of multiple reflections and refractions. The important criterion for the particles is that they be transparent to the wavelength of incident radiation used for detection.

The transparency of the particles used to incident radiation used for assay must be such that differences in transmission or excitation must be readily measurable at the desired wavelength. This degree of transparency is hereinafter referred as "substantially transparent". This is the essential criterion for the particles to be used in each assay. Of course, the necessary degree of transparency is decreased if the sample is spread into an optically thin layer, or the intensity of the light source is increased.

Many different types of particles can be used, including carbohydrates, polystyrene or other plastics, and the like. The most commonly used particles are carbohydrates such as dextran, agarose, agar, deacetylated chitin, or starch. Agarose and dextran are preferred. These particles may form free-standing gels or may require crosslinking with crosslinkers such as epichlorohydrin or glutaraldehyde.

Conveniently, the particles are in the form of beads which are roughly spherical, having a diameter of from about 20 to about 400 microns. Porous particles are preferred. The porosity of the particles may range from about 10% to about 99% of the volume of the particles.

Of course, when porous particles are used, some of the liquid in the medium is absorbed into the interior of the particles, in some cases altering the transparency of the particles. In this case, the liquid medium used should be such that, at the end point of absorption, differences in transmission or excitation of incident light are readily measurable at the desired wavelength.

As the particles absorb solvent, the refractive index of the particles changes. As the particles absorb solvent, their

refractive index decreases as compared to the solvent. As the particles absorb solvent, and swell, their refractive index approaches that of the solvent. As shown above with respect to Sephadex particles, each particle matrix possesses its own degree of swelling.

Immobilization

The affinity reagent is immobilized directly or indirectly, covalently or noncovalently, on the particle. More than one affinity reagent may be immobilized on the same or different particles. Among the compounds that can be used to immobilize the affinity reagent to the particles are para-nitrophenyl chloroformate, cyanogen bromide, glutaraldehyde, epoxy groups, divinyl sulfone, epichlorohydrin, and others, provided the crosslinking agent is suitable for the specific application.

Incubation

The purpose of incubation is to expose the particles to the reagent and sample until an equilibrium is reached for the attachment of an analyte to the particle. Efficacy of continuous mixing determines the speed of reaching equilibrium. Turbulence may be supplied to the system by tumbling, shaking or passing bubbles through the reagent-sample mix. In the case of bubbles, this type of mixing may be begun before sample is added to the system. The optimum incubation period is related to the degree of specific affinity of the reagent for the analyte, as well as to the quantities of reagent and analyte present. Typically, the incubation is for about five to ten minutes.

Fractionation

After incubation, the mixture is fractionated within a single reaction vessel into a particle-rich fraction and a substantially particle-free fraction. The substantially particle-free fraction is sufficiently free of particles so that any particles in the substantially particle-free fraction do not detectably refract light transmitted through this particle-free

fraction. The fractionation may be effected merely by permitting the particles to settle to the bottom of the reaction vessel under the influence of gravity, or the mixture may be centrifuged to hasten the fractionation.

A typical fractionation time under normal gravity is about ten to fifteen minutes. Alternatively, a one to three minute centrifugation at about 500 x G provides immediate constant readings which compare favorably to 10-15 minutes of settling.

As a further alternative, the particles may be collected on a (polypropylene) frit that transmits liquid but not particles with very slight pressure. Here the reading becomes constant almost immediately in both particle and liquid region, where the liquid has passed through the frit. The disadvantage of this alternative is the slight increase in cost of providing the frit.

Apparatus

Apparatus for use in the present invention are shown in Figures 1 and 2. The apparatus (2) comprises a reaction chamber (4) having a cap (6) which may be opened but which is liquid tight when closed. The cap (6) may be attached by a loop of plastic material to the reaction chamber (4). The bottom of the reaction chamber (4) is cone shaped and is connected to the measuring container (8) so that the hollow insides are continuous. A plug (10) separates the inside of the two chambers during storage and when sample (12) is first inserted into the reaction chamber (4). The reaction chamber (4) is conveniently made of a lightweight unbreakable plastic material such as polypropylene. However, the exact composition of the material of the reaction vessel is not important, as long as the material can be molded to proper shape and is chemically inert to the reactants.

The optical measuring container (8) may be made of any optically transparent material such as plastic or glass. Glass is preferred, unless the plastic is of assured optical quality. The bore (14) may be round or may have parallel sides, although a round bore is preferred. The bore is generally between about

1 mm and 3 mm, but more preferably, 1.2 to 2 mm. The most preferred bores are about 1.6 mm in diameter. Of course, the ultimate bore size is determined by the refractile characteristics of the particles in the liquid. Where the refractive index of the particles closely matches the refractive index of the solution, the preferred bore may be greater than 1.6 mm. The bore (14) must be of known geometry for the length of the measuring container (8).

A mixing means, here portrayed as a small plastic air tube (18) providing positive pressure to the reaction chamber (4), supplies bubbles (20) to the bottom of the reaction suspension (22) during the mixing time. As the bubbles (20) rise, turbulence is created, providing the required mixing action. At the end of the mixing period, the plug (10) is pulled by means of an attached string (24), thus providing continuity between the reaction chamber (4) and the bore (14) of the measuring chamber (8). Liquid suspension (22) then settles into the measuring chamber and is stopped from exiting by a tightly fitting frit (26) inserted part way on the length of the measuring container (8). The frit (26) retains the particles (28) but permits passage of liquid (30). The precise additional pressure required to initiate flow of liquid (30) past the frit (26) and to stop before exiting the bottom (32) of the measuring chamber (8), is supplied by closing the cap (6).

To ensure that no liquid leaks from the bottom (32) of the capillary (8), a closure (34) may be provided. Optionally, the reaction chamber (4) can be separated from the measuring container (8).

The optical paths for the particle-rich fraction (36) and for the particle-free solution (38) are defined at positions along the measuring container by the position of the frit.

Alternatively, a source of positive pressure (18) may be supplied through the measuring tube (8) which is opened to the mixing chamber before sample is added therefor. Thus, bubbles (20) are supplied to the mixing chamber (4) through the measuring tube (8). At the end of the mixing period, the

pressure is reversed and both particles and solution settle into the measuring tube (8).

Alternatively, the reaction chamber (4) and the optical measuring container (8) may be separated from the inception of the procedure, and means for transferring a portion of both particles and solution is provided. Such means may be a precisely metered negative pressure displacement device, such as a pipette. In this case, a closure (34) for the bottom of the optical measuring container (8) is required.

Optical Reading Methods and Apparatus

One means for reading the results obtained using the method of the present invention is a narrow slit transmission of white light through a capillary with a slit and a narrow range of filters before an optical sensor (photodiode). The Becton Dickinson QBC Autoread is one example of this type of device, and Example 1 provides data obtained with this instrument.

In another type of device narrow slit excitation light, white light is the source with a narrow band filter, while the fluorescent analysis is with broad band light filters. This is the standard BD Autoread with no modification of filters.

In a broad slit white light source which scans multiple (e.g, 12) parallel capillaries at one reading, the white light source is wider than the capillary and the capillaries are each laid into a slot which limits transmission of light around the capillary. This is a microtiter plate reader adapted to read capillaries. Alternatively, a reader can be used which reads fluorescence instead of transmission.

In another type of reader, the capillary is surrounded by a cylindrical lens. The lens accepts a broad, parallel light beam which is focused onto the capillary through which the light is transmitted. As light exits, the light is spread again into a broad parallel beam. The light for this assembly is two narrow band light emitting diodes, 50, 51 of which one (50) provides light in the 560 nm range and the other (51) in the 635 nm range. This system is manually positioned to read first the

particle-rich zone and then the particle-free zone. An alternate optical path is shown at 62.

An extension of this system is a design with two parallel optical trains 60 which read simultaneously as the bottom of the capillary triggers a microswitch upon insertion. In this design, a microprocessor controls the on-off switching of light emitting diodes and computes the results. Thus, results are obtained immediately upon inserting and removing the capillary.

Another extension of the above-described light emitting diode instrument has a single pair of light emitting diodes and the capillary is passed rapidly through the hollow cylindrical lens by gravity as the microprocessor sorts out data from particle and solution phases.

Absorbance

Because compounds have individual absorbances, the wavelength of incident light used for assays according to the present invention can be chosen specifically for the analyte or analytes sought to be detected.

One example of absorbances that can be used is for hemoglobin. Hemoglobin absorbs strongly in the "blue" (400-420 nm) and "green (540-570 nm) regions of the spectrum. While any wavelength from 400 to 590 nm can be used for detecting hemoglobin, 560 nm is preferred. On the other hand, hemoglobin does not significantly absorb light in the "red" region (630-690 nm). Any wavelength in this "red" region may thus be used. Any absorbance at this red wavelength will be due to unintended variations in the optical path, and thus is not due to the presence of hemoglobin. By computation, the transmission in the "red" at each point is set to 100%, and the transmission, for example at 560 nm, is used to determine the absorbance of the particle-free zone and the particle-rich zone at each point. This calculated absorbance has been corrected for minor light scattering variations in transmission. If the particles used are of a certain size, color, or opacity, depending upon the wavelength used, there may still remain a residual uncorrected variation. This residual variation can be easily determined by scanning a capillary that contains all of the components except

hemoglobin. The difference in the calculated absorbance of the particles to the supernatant regions is the residual "correction factor" and is incorporated into the final algorithm for calculation % glycosylated hemoglobin.

Of course, the absorbances of a great many compounds are known, and one skilled in the art can readily determine at what wavelengths assays should be conducted for any particular analyte in order to optimize the results. Many handbooks and textbooks give absorption peaks for a number of compounds, e.g., Handbook of Fluorescent Probes and Research Chemicals, 5th edition, 1991-1994, Haughland, ed., Molecular Probes, Inc., Eugene, 1992. This particular handbook shows absorption peaks for reagents as well as absorptions for may chemicals available as fluorescent probes.

The present invention requires that the particles be substantially transparent to the wavelength of radiation used or detection. The amount of the analyte of interest in the particle-rich zone may then be determined optically by correlating the absorbance of the particle-rich zone, at a wavelength characteristic of the analyte, with a suitable standard. In contrast to several of the procedures mentions previously, there is no need to physically remove the unbound fraction, or to collect the various fractions in different containers. Of course, with the opaque particles typically used in the art, absorbance measurements are not practical.

Types of Tests

A great variety of tests can be conducted using the present invention. Reading of the solutions and particles can be effected by light absorption, refractive light loss, fluorescence, and fluorescence quenching, for example. Readings may be taken of the absorbing analyte, the fluorescing analyte, an absorbing intermediate such as a linker, fluorescence of a linker, absorbance of a small molecule, and refraction of a large molecule.

In one preferred embodiment, the absorption of light is measured. It has now been found that the imprecisions and inaccuracies inherent in optical measurement through particles

can be improved. For example, the region of the particles which has adsorbed the attracted constituent also contains some of the solution, both between the particles and within each particle. This solution is herein defined as the Free Volume space. This Free Volume space may be determined experimentally, and is constant for each procedure. This is particularly true in the case of porous particles.

The dispersion of light by the particles is influenced by the solid mass contained in the particles. The solid mass increases as more constituent is adsorbed onto the particles. This dispersion is taken into account as an additional adjustment in the computation. The constant for this adjustment is determined experimentally.

The dispersion of light just described also provides a means for directly measuring the mass of constituent independent of a specific absorbing wavelength. One embodiment of the invention uses this dispersion as the end point measurement. This technique is especially useful when the constituent to be measured has no color contrast which can be used with simple instruments. In measuring glycoalbumin, mass measurement may be used to replace an ultraviolet absorbance measurement.

Inserting a fluorescent compound such as 8-hydroxy-1,3,5-pyrene trisulfonate (HPT) into the allosteric pocket of the hemoglobin molecule causes quenching of the strong fluorescence of HPT. This quenching has also been described by MacQuarrie and Gibson in J. Biol. Chem. 274 (18): 5686-5694. HPT fluorescence quenching by hemoglobin is quantitative, and has been found to be independent of the glycation of hemoglobin. It was also surprisingly discovered that the fluorescence decrease due to competitive absorption of light by hemoglobin provides a sensing means which is independent of quenching due to ligand binding, provided that either excitation or fluorescence wavelengths, or both, of the fluorochrome overlap an absorption wavelength of hemoglobin.

In one embodiment of the present invention, quenching of HPT fluorescence is used as the means for measuring total hemoglobin. The further reduction of fluorescence, especially in the region of the particles, is due to reduced transmission

of the exciting and emitted light through the hemoglobin adsorbed to the particulate. In one embodiment of the invention, this reduced transmission is measured by the reduced fluorescence and is then recalculated as an optical absorbance (optical density) resulting from the presence of hemoglobin.

The following examples are provided to illustrate the present invention, and are not to be construed as limitations.

EMBODIMENT 1: GLYCOHEMOGLOBIN BY OPTICAL ABSORPTION (EXAMPLES 1-3)

Reagent A was prepared by raising the pH of a 0.27 M ammonium acetate buffer which contains 0.05 M magnesium chloride to pH 8.0 by adding concentrated ammonium hydroxide. The resin used was aminophenyl boronate attached through a spacer arm to crosslinked beaded 6% agarose gel. The resin was washed with water, and was finally washed with Reagent A. A precise proportion of 1 part settled resin to 9 parts Reagent A was transferred to reaction vessels which were closed by caps for storage. The total fill was approximately 450 μL for each reaction vessel.

A volume measuring capillary was used to transfer approximately 10 microliters of sample to the reaction vessel. Hemolysin agent may be introduced into this capillary prior to use where appropriate.

The optical measuring capillary was a round capillary, 75 mm in length, with an inside diameter of 1.6 m. A closure was supplied for one end of the capillary.

Approximately 10 microliters of mixed whole blood derived from a finger puncture or from a tube of blood using EDTA coagulant or a purified hemoglobin standard solution was taken up into the volume measuring capillary where the hemolysin agent lysed the red blood cells. The blood mixed with hemolysin agent was transferred to a separate reaction vessel which contained Reagent A. The reaction vessel was then closed and mixed by rotation for approximately five minutes.

At the end of the mixing time, a portion of the reaction mixture containing both resin and buffer was transferred to the optical measuring capillary, which was then closed at its bottom

and gently tapped to eliminate bubbles. The capillary was made to stand upright so that the resin settled to the bottom. The settling may be accelerated by centrifugation; however, the resin reached a constant compacting without centrifugation in about 10 minutes.

Optical transmission readings were taken at 560 nm and 630 nm at both the settled resin portion and in the region which contains solution substantially free of resin. These measurements may conveniently be made with a microtiter plate reader using a plate adaptor to hold twelve parallel capillaries. Each capillary occupies eight well positions of an 8 x 12 or 96 well plate.

To complete the calculations, measurements are made on a blank reagent capillary to which no sample has been added, and another reagent capillary containing a hemoglobin sample with no glycohemoglobin. The measurements are merely a calibration to provide constants to the calculation, and need not be performed concurrently with each sample.

Calculations

For convenience, the calculations in this example were made on the measured optical density output of a microtiter plate reader. Of course, it will be obvious to those skilled in the art that the initial measurements are electrical, such as in millivolts, which are then converted to optical density values by the instruments.

The reading of the solution portion of the blank is defined as 100% transmission or the equivalent, 0 absorbance (OD), when the reading at 630 nm is subtracted from the reading at 560 nm.

The reading through the resin portion of the blank was likewise taken and was subtracted from every other reading though resin as a correction for differential scattering of the green light versus the red light.

Free volume (FV) of the resin was calculated from the two additional readings made in the 0 glycohemoglobin capillary:

$$FV = \frac{OD\ resin}{OD\ solution}$$

The Free volume is a proportion, representing the space in the resin occupied by and continuous with the solution. This value must always be less than 1 (100%), because part of the space is occupied by the solid portion of the resin and its hydration, from which molecules like hemoglobin are excluded. FV is often in the range of 0.7 to 0.98.

The glycohemoglobin attached to the resin is calculated from the sample measurement:

GR= OD resin - (FV x OD solution)

and the proportion of glycohemoglobin to total hemoglobin is calculated from the sample measurements as

GHb = GR/[OD resin + (9 x OD solution)]

Finally, an adjustment is made for refractive light losses through the resin at the nonabsorbing wavelength (630 nm). This adjustment is characteristic for each type of measuring instrument, and depends primarily on the numerical aperture of the light collection lens in front of the light sensor. Conversion from proportion to percent is also accomplished in this final calculation:

GHb %= 100 x GHb - (A x GR)

A is the adjustment, which is usually a negative number. Thus, calculation of GHb % without applying A as an adjustment usually yields an underestimate for GHb %.

Example 1

130 whole blood samples taken from diabetic patients as well as nondiabetic controls were subjected to the above procedure and calculation. The results are shown in Figure 3 as compared to a standard commercial method of testing for glycohemoglobin (Helena Laboratory Columnmate).

Example 2

The same samples and capillaries as in Example 1 were again read in a modified Becton Dickinson QBC Autoread (7 Loveton Circle, Sparks, MD), where the light source, originally a red LED, was replaced by a halogen tungsten lamp, and filters for 560 nm and 630 nm were supplied.

The results obtained as compared to a standard procedure are shown in Figure 4.

Example 3

The same samples and capillaries as in Example 1 were read in an engineering prototype, in which the light source and filter were replaced by light emitting diodes supplying light at 560 and 630 nm. The on-off timing was translated to voltage in a single measuring photodiode. Absorbance at 560 nm and 630 nm was determined, and the computations were as in Example 1. In this prototype, the optical measuring capillary was manually positioned first with the resin and then with the free solution in the light path to obtain the measurement results.

The results as compared to a standard procedure are shown in Figure 5.

EMBODIMENT 2 (EXAMPLES 4-6)

Reagent B was prepared. Reagent B was identical to Reagent A of Embodiment 1 except for the addition of 111 Mm 8 hydroxy-1,3,5-pyrene trisulfonate to the ammonium acetate/magnesium chloride buffer, pH 8.0

Samples were prepared for optical measurement as in Embodiment 1.

Optical Measurement

Fluorescence excitation was accomplished with a white light source supplied with a blue filter transmitting only in the range of 460 to 490 nm.

Fluorescent light was collected and measured after passage through a green filter that transmitted light between 530 and 650 nm and specifically rejected the excitation wavelengths.

Calculations

For the purpose of simple calculations, the decrease in fluorescence observed in the sample compared to a blank with no hemoglobin is treated as absorbance.

$$\text{OD solution} = \log \frac{\text{(FL solution blank)}}{\text{(FL solution sample)}}$$

$$\text{OD particulate} = \log \frac{\text{(FL sample solution x B)}}{\text{(FL sample resin)}}$$

B is an adjustment required for differences in collection of fluorescence light in the region of the resin as compared to the region of the solution. B is dependent on the numerical aperture of the light collecting lens in front of the sensor and differs for each instrument design as well as for each type of resin used.

As can be seen in the examples described below, OD solution yields a result which closely correlates to the total hemoglobin in the sample. Likewise, OD resin yields a result that correlates with glycohemoglobin in the resin, but this result is not independent of the total hemoglobin in the sample. Therefore, the computation of percent glycohemoglobin in the sample is:

GHb% = 100 x S(OD resin - C x OD solution x OD resin)

where S is a slope adjustment of a regression equation and C is an adjustment related to the free volume as explained in Embodiment 1.

Example 4:

Hemoglobin Recovery

Purified hemoglobin with varying proportions of glycohemoglobin was prepared. A standardized volume of each of three dilutions of the prepared hemoglobins was put into a reaction vessel using Reagent B. Thereafter, the procedure of Example 1 was followed, except that fluorescent measurements were made according to Embodiment 2.

The results are expressed as OD solution, and are given in Figure 6, compared to OD units measured on an independent spectrophotometer at 410 nm on the diluted samples. Absolute measure of hemoglobin requires an absolute standard which was not tested. Therefore, the relationship is provided solely as

a comparison of the absorbencies. These can, however, be easily standardized by one skilled in this art.

It can be seen from Figure 6 that the correlation between hemoglobin with a standard spectrophotometric measurement and the fluorescence measurement is very high. The regression equation may therefore be used to provide results at intermediate values.

Similar experiments were performed in a reagent excluding the resin. The resin is not required when only total hemoglobin is to be determined. The results provided equally good correlation.

Example 5

Glycohemoglobin Recovery

The procedure of Example 4 was followed exactly, and fluorescent measurements were made in the solution and the resin region according to the second embodiment.

The results are given in Figure 7. The measurement results are expressed as proportions, which may be translated to glycohemoglobin percent by adjustment S. Comparison measurements were made with a standard commercially available glycohemoglobin kit, Pierce Glycogel II.

It can be seen that the results correlate very well over the whole range of expected clinical values. Therefore, the regression equation may be used to extrapolate results from measurements of unknown samples.

Example 6

The procedure and measurements of Example 5 were followed exactly. However, calculations of results for glycohemoglobin were derived directly from the voltage measurement of the fluorescence sensor made in the region of the solution and the region of the particles. A simple ratio is derived as follows:

$$\text{Ratio} = \frac{\text{Volts (fluorescence) particulates}}{\text{Volts (fluorescence) solution}}$$

The results are shown in Figure 8, using the same comparison as in Figure 7. It can be seen that this simple

ratio provides excellent correlation with a reference method over the whole range of clinical values. Therefore, the regression equation may be used to extrapolate results from measurements of unknown samples.

EMBODIMENT 3 (EXAMPLE 7)

The reagent used in this embodiment was Reagent B.

Optical Measurement

In addition to measuring fluorescence of the solution as in Embodiment 2, optical light loss was measured through the capillary at 630 nm, using a red LED at about 630 nm as the source. The readings were compared with a blank and absorbencies (OD) were computed.

By subtracting the OD solution from the OD resin, an estimate of glycohemoglobin attached to the resin was made. This estimate was based on the increase of refractive index of the resin due to its increased mass when glycohemoglobin was attached to the resin.

Calculations

The total hemoglobin obtained by fluorescence measurement was used to normalize absorbance at 630 nm.

Example 7

The identical tubes used in Example 5 were used and measured according to Embodiment 3.

The results are given in Figure 9 with the same comparison method to Pierce Glycogel II. It can be seen that the correlation between the reference method and the experimental method is excellent, and therefore, the regression equation may be used to compute glycohemoglobin of samples where the glycohemoglobin is not known.

EMBODIMENT 4 (EXAMPLES 8-10)

A dimer of aminophenyl boronic acid was synthesized, in which a spacer arm of at least six carbon atoms was between the two amino groups.

An acid reducing Schiff reagent was prepared from meta aminophenyl boronic acid by reacting the meta aminophenyl boronic acid overnight with 0.5 N hydrochloric acid and 1% by weight of sodium metabisulfite. To the Schiff reagent was next added repeatedly small amounts of 0.1% glutaraldehyde in 0.1 N HCl until, one hour after an addition, the test for aldehyde using a basic fuchsin Schiff reagent was positive. At this point, it was assumed that the m-aminophenyl boronic acid had been used up and a preponderance of dimer had been formed.

By adding an excess of disodium phosphate, the pH was brought to 8 and sodium borohydride was added, thus providing a stable amino compound with a preponderance of dimers.

Salts were removed from this dimer preparation and the desired dimer was concentrated by mixing with a dextran solid support, such as Sephadex G 25. The compounds containing boronic acid adhered to the dextran, while the salts could be eluted. The desired dimer may then be eluted in various solutions, such as an acidified methanol.

Reagent C

The buffer of Reagent 1, Ph 8.0 ammonium acetate/magnesium chloride buffer was mixed with a dextran gel, such as Sephadex G 100, in proportions of 9:1.

Volume Measuring Capillary

As in Embodiment 1, the capillary contained both lysing agent and the dimer of aminophenyl boronic acid, preferably in dried form.

The procedure was identical to Embodiments 1 and 2.Both the fluorescence and absorbance measuring methods can be used.

The present invention thus provides methods for measuring a variety of substances in a sample. In each case, the optical contrast to be measured must be matched to the compounds to be measured. Thus, a general method of measuring two components in a sample is implemented with an affinity particulate and a solution without the requirement for eluting the attached constituent from the particles.

Example 8

Blood Group Detection with Lectins

This is a competitive binding assay. An analog to the blood group substance sought to be detected is attached to the particles on a molecular arm long enough to make the analog accessible from solution. A lectin specific for the same blood group substance, such as concanavalin A for mannose, is added to the particles. This lectin is optionally made fluorescent, for example with fluorescein isothio-cyanate. When a sample containing variable amounts of the true blood group substance is added, the fluorescing lectin distributes itself competitively between the solution and the particles. If, for example, there is no blood group substance in the sample, all of the fluorescing lectin will be adsorbed onto the particles. The proportions of particles and lectin are arranged so that the amount of blood group substance in a sample may be detected or measured.

This test can be used for blood typing as well as for detecting contaminating blood types in a sample. Fetal red cells in maternal blood is an example of such as contamination.

A partial list of pairs of lectin and blood type which can be used is as follows:

1. Griffonia simplifolia I-A_4 N-Acetyl galactosamine A
2. Griffonia simplifolia I-B_4 Galactose B
3. Laburnum alpinum I Oligo N-acetyl glucosamine O

Example 9

Four Wavelength Method

Both hemoglobin and chlorophyll are prophyrin compounds. Although they have in common one absorption peak at around 410 nm, the "Sorret band", their absorption bands are otherwise different and do not overlap.

Beads of boronic acid affinity agarose were saturated with chlorophyll A. The saturated beads have optical characteristics similar to chlorophyll a in solution in that they absorb at 400-450 nm, they are opaque from 650 to 680 nm, and they transmit light well between 450 and 650 nm. These beads retain their specific chemical affinity character.

Applying appropriate correction factors applied to adjust for incomplete transmission at 450-650 nm and for light scatter effects, it is possible to calculate % glycohemoglobin making four wavelength measurements on individual microtiter plate wells containing buffer at Ph 8.0, lysing agent, a small portion of whole blood, and affinity beads containing chlorophyll a which partially obscure the light path.

The following table shows the effect of incident light at a variety of wavelengths.

TABLE 2

Wavelength	Description
670 nm	100% transmission of partially obscure light path (Correction factor for 410 nm)
410 nm	Hemoglobin absorption of partially obscure light path
410-670 nm	Non-glyco hemoglobin (GHb hidden in beads)
630 nm	100% transmission, including light through beads
560 nm	Hemoglobin absorption through solution and beads
560-630 nm	Total hemoglobin in solution and in beads.

With appropriate constants and corrections for degrees of transparency in various wavelength readings, all of which may be determined experimentally, the percent glycohemoglobin in the sample is:

$$GHB\% = \frac{(Abs\ 560 - Abs\ 630) - (Abs\ 410 - Abs\ 670)}{(Abs\ 560 - Abs\ 630)}$$

With this method, it is possible to perform specific hemoglobin analysis, such as glycohemoglobin, in a single well of a microtiter plate reader. This is yet a further improvement in efficiency over the capillary tube method.

Example 10

Boronated affinity methods are conventionally used to measure glycated proteins (hemoglobins and plasma proteins). These proteins differ from non-glycated proteins by the attachment of at least one sugar moiety to the protein at

various binding sites by means of a ketoamine bond. Glyco-hemoglobin thus contains 1,2-cis-diol groups not found in non-glycated hemoglobin. These diol groups provide the basis for separation of glycated and non-glycated components by boronate affinity chromatography. In boronate affinity chromatography, a boronate such as phenylboronic acid is bonded to the surface of a column support. When a solution of proteins (hemolysate or diluted plasma) is passed through the column, the glycated component is retained by complexing of the diol groups with the boronate. After the unretained non-glycated component elutes from the column, the glycated component is eluted from the column with a reagent that displaces it from the boronate.

Glycohemoglobin measurement by boronate affinity methods is free from many interferences such as hemoglobin variants, non-glycation modifications and storage-related hemichromes. Boronate affinity methods require no sample pretreatment to remove the labile (aldimine or Schiff bases) components, since only stable (ketoamine-linked) glyco-hemoglobin is retained by the boronate. Compared to ion-exchange techniques, affinity separation is also less sensitive to quantitative errors caused by minor fluctuations in reagent pH and ionic strength. Changes in ionic strength have minimal effect on affinity values.

The method of the present invention combined the advantages of the interference-free boronate affinity separation with the convenience of being able to read through the gel formed with the substantially transparent particles. After glycated hemoglobin attaches to the boronate gel, the separation of glycated from non-glycated hemoglobin is effected by gravity in a single capillary tube which is read directly on a reading device (spectrophotometer, etc.), thus eliminating the need for multiple buffer separations and readings as in other methods. To conduct the tests described below, samples were introduced into reaction vessels. Each reaction vessel containing 350 microliters of a 10% v/v gelphosphate buffer mixture. The gel was immobilized m-aminophenol boronic acid on crosslinked 6% agarose. The pH 8.0 phosphate buffer contained 0.02% sodium azide as a stabilizer and 0.06% Triton S 100(R) as a lysing agent.

Whole blood samples were introduced into the reaction vessels and the vessels shaken to dislodge any gel from the bottom and completely lyse the red cells. The vessels were placed onto a rotator and mixed for a minimum of 10 minutes. The gel was permitted to settle in the reaction vessel. The vessel may be centrifuged for 30 seconds at approximately 8000 rpm's to help settle the gel. Then, 100 microliters were slowly withdrawn into the capillary of the vessel. The end of the capillary was sealed by pressing the end into a sealing compound. The sample was spun in a Microhematocrit centrifuge at approximately 13,000 g's for one minute. The sample was stable and may be read up to five hours later provided the gel layer was not disturbed. The capillary was then placed into the reading device and the optical densities of the clear supernatant and the gel were determined at both 560 nm $\pm$ 2 nm and 630 nm $\pm$ 2 nm.

Calculations

$$\text{Observed value} = \frac{100\,[(G-B_g) - (0.94 * (S-B_s))]}{9\,(S-B_s) + (G-B_g)}$$

Where:

Each optical density reading (OD) in the calculation is obtained by subtracting the OD at 630 nm from the OD at 560 nm.

G = OD of the sample read through the gel

B_g = OD of the bland read through the gel

B_s = OD of the bland read through the solution

S = OD of the sample read through the clear solution

0.94= free volume constant

9 - dilution factor

$$\% \text{ GHb} = \frac{O_s - bc}{M_c}$$

Where:

O_s = observed value of the sample

$$M_c = \frac{O_H - O_L}{P_H - P_L}$$

O_H = observed value of the High calibrator

O_L = Observed vale of the Low calibrator

P_H = the assigned value of the High calibrator

P_L = the assigned value of the Low calibrator

$B_c = O_H - M_c * P_H$

A series of 56 men and women with no known diseases provided samples for determination of percent glycated hemoglobin. The results are summarized in Figure A

A series of 74 men and women with known diabetes provided samples for determination of percent glycated hemoglobin. The results are summarized in Figure B.

To measure specific performance characteristics of the assay of the present invention, whole blood from a diabetic patient (GHb = 25/8%) was mixed in increasing proportion with whole blood from a non-diabetic subject (GHb = 4.7%) and expected GHb levels were calculated for each mixture. Table 3 compares the calculated GHb with the assay of the present invention for each mixture:

RATIO LOW/HIGH	EXPECTED VALUE	OBSERVED VALUE	RECOVERY
(low)	4.7%	4.5%	96.2%
4/1		8.9%	7.9% 88.8%
3/2		13.1%	13.9%105.7%
2/3		17.4%	17.8%102.7%
1/4		21.6%	23.3%107.8%
(high)	25.8%	26.1%	101.2%

Effect of Protein Concentration

To test the effect of normal variations in hemoglobin on the results of the assay, one normal and one diabetic, whole blood specimens were assayed using various amounts of sample ranging from 5.0 microliter to 20.0 microliter (recommended is 10 microliter). Table 4 shows the results of an assay conducted as in Example 10:

TABLE 4

EFFECT OF Hb CONCENTRATION

Sample Size	Normal	Diabetic
5.0µl	4.3%	22.3%
7.5µl	5.7%	22.6%
10.0µl	3.8%	21.3%
12.5µl	4.4%	21.7%
15.0µl	4.2%	20.9%
17.5µl	4.2%	20.9%
20.0µl	4.2%	20.6%

Effect of Labile Components

It is well known that labile glycated components do not interfere with the measurement of GHb in boronate affinity methods (Fluckiger et al., Diabetes 33: 773-76 (1984); Baynes et al., Diabetes Care 7:6 (1984); Gould et al., Ann. Clin. Biochem. 21: 16-21 (1984); Johnson et al., Clin. Chem. Acta 127: 87-95 (1982).

Corrections

WITHIN-RUN PRECISION

Whole blood samples from a normal subject and a diabetic subject were assayed 20 times within the same run, with the results shown in Table 5:

TABLE 5

WITHIN-RUN PRECISION

Normal	High
n = 20	n = 20
x = 5.1%	x = 34.9%
SD = 0.17	SD = 0.64
CV = 3,33%	CV = 1.83%

Run-to-run Precision

Samples with low and high glycated hemoglobin levels were assayed repeatedly over a two day period with the results shown in Table 6:

TABLE 6

RUN-TO-RUN PRECISION

Normal	High
n = 20	n = 18
x = 6.3%	x = 25.5%%
SD = 0.38%	SD = 1.30
CV = 6.1%	CV = 5.1

Correlation with other methods

Whole blood samples from twenty one individuals, including non-diabetic and diabetic patient, were measured by the method of Example 10, and the results were compared to five other commercially-available methods: GHb by phenylboronate affinity (PBA) minicolumn, HbA_{1c} by ion-exchange high-performance liquid chromatography (IEX-=HPLC) and GHb by phenylboronate affinity high-performance liquid chromatography (PBA-HPLC). Table 7 shows the results of a linear regression analysis

TABLE 7

Assay	Method	Lin. Regress. Equation	r
GHb	PBA minicolumn	y = .48x - 0.76	0.940
HbA_{1c}	IEX-HPLC	y = 0.53x - 2.20	0.988
GHb	PBA-HPLC	y = 1.022x - (-0.347)	0.996

Another use of the present invention is for detection of circulating specific antibodies in plasma. In this case, the particles contain the opposite of the antigen-antibody pair, while a fluorescing antigen is dissolved in the solution. The assay is a competitive assay, and the proportion of fluorescing antigen which remains in solution relates to the amount of antibody in the plasma.

This variation of the assay can be used to test for antibodies to hepatitis virus and AIDS virus.

Circulating antigen can be tested for by attaching antigen to the particles and using antibody to the analyte as the fluorescing solution.

The present invention can also be used as an analog to gel filtration chromatography. In this example, a small molecule is labelled with a fluorescent marker, such as fluorescein isothiocyanate (FITC). The particles are constructed so that the small molecule with its fluorescent label is able to penetrate and diffuse freely, both within the particles and in the solution external to the particles. The analyte is a large molecule which is not able to penetrate the particles. Additionally, the analyte and the small, labelled molecule will combine if they are present in the same solution. The combined analyte-small molecule is also excluded from diffusion into the particles. Distribution of fluorescence between particles and solution provides a means for quantifying the specific large molecule.

This method can be used to measure soluble starch. The large molecule, with the dansyl derivative of m-aminophenyl boronic acid, sold under the name of Fluorobora I by Polysciences, Inc. uses particles of crosslinked 6% agarose, such as Sepharose 6CL, from Pharmacia, Inc., which excludes molecules whose molecular weight is greater than 4×10^6. The small and large molecule combination are with small fragment DNA probes, which hybridize with high molecular weight DNA in a sample solution.

Total antibody gamma globulin is measured by attaching Protein A to a particle such as 4% crosslinked beaded agarose. The attachment of gamma globulin to this particle is sufficient to cause a change in the refractive index of the particles, and thereby provide a loss of light which is proportional to the amount of gamma globulin attached to the particles.

The affinity reagent attached to the particles, such as Protein A, may be attached by a variety of active compounds, including para-nitrophenyl chloroformate, cyanogen bromide, glutaraldehyde, epoxy groups, divinyl sulfone, epichlorohydrin,

and others. The only requirement is that the crosslinking agent be suitable for the specific application.

The method and apparatus of the present invention permit measurement of analytes that can be preferentially bound to particles while in solution. This method does not require separation of bound and unbound fractions into separate containers, thus obviating the need for extra steps and possible loss of analyte.

Because the particles used are substantially transparent to the liquid medium used at the wavelengths of radiation used, the method includes reading optically through a particle-rich zone as well as through a particle-free zone. Because the method includes reading through both the particle- rich and the particle- free zone, the method automatically adjusts arithmetically for both optical and liquid distribution anomalies that would otherwise cause errors in the results. Prior methods required correction of multiple features, and thus were not able to provide simple automatic correction for the presence of extraneous particles in the particle-free zone or for the optical properties of the liquid medium.

The present invention is not limited to the types of particles that can be used, as long as the particles at the time of detection are transparent or translucent to the wavelength of radiation used for detection.

Using affinity particles greatly diminishes the cost of assays conducted as compared to conventional batch or chromatographic methods. The assays of the present invention require the use of a much smaller portion of particles of assay as compared to conventional chromatographic techniques. Since the particles are generally the most expensive components of these types of assays, the cost savings may be considerable.

Because the method of the present invention requires fewer liquid manipulation steps, there is greater precision with reduced labor costs as compared to the conventional chromatographic methods.

The relative proportion of analyte is adjusted by concentrating the analyte present into a smaller volume for optical reading. This feature of the present invention improves

precision of the assay, and permits assay of lower concentrations of analyte than had heretofore been possible.

The device used to detect the particle-free and particle-rich zones are not critical to the present invention. Although absorption readings may differ from device to device, when using the same capillaries, the correlation of the analyte in the samples was better than r=0.999.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

WE CLAIM:

1. A method for measuring an analyte in a sample comprising adding substantially transparent particles to a sample in solution or suspension, said particles having an affinity for said analyte;

incubating the particles with the sample;

fractionating the particles from the solution or suspension to form a particle-rich fraction and a substantially particle-free fraction;

optically reading the particle-rich fraction at a first and a second wavelength;

optically reading the substantially particle-free fraction at a first and a second wavelength;

correlating the readings through the particle-rich fraction and the substantially particle-free fraction to obtain a quantitative determination of the analyte originally present in the sample.

2. The method according to claim 1 wherein the analyte consists of a first constituent and a second constituent.

3. The method according to claim 1 wherein the particles are attached to an affinity reagent.

4. The method according to claim 3 wherein the affinity reagent is selected from the group consisting of carbohydrates, antibodies, antigens, enzymes, enzyme substrates, and enzyme inhibitors.

5. The method according to claim 1 wherein said particles are selected from the group consisting of dextran, agar, deacetlytated chitin, and starch.

6. The method according to claim 2 wherein said first constituent is glycosylated hemoglobin.

7. The method according to claim 6 wherein said second constituent is unglycosylated hemoglobin.

8. The method according to claim 1 wherein said solution or suspension is fractionated by centrifugation.

9. The method according to claim 1 wherein said fractionation is effected by permitting the particles to settle under the influence of gravity.

10. The method according to claim 1 wherein said fractionation is effected by collecting said particles on a frit.

11. A kit for measuring an analyte in a sample comprising a reaction vessel containing a reagent comprising partially transparent particles which adsorb the analyte preferentiality; and an optical measuring container which provides a first path for light and a second path for light for optical measurement.

12. The kit according to claim 11 further including means for providing turbulence to said reaction vessel.

13. Apparatus for measuring an analyte in a sample in a solution of suspension of said sample comprising a reaction chamber, a measuring chamber, and a first optical path and a second optical path in said measuring chamber.

14. The apparatus according to claim 13 further including a means for mixing said sample with at least one reagent in said reaction chamber.

15. The apparatus according to claim 14 wherein said means for mixing comprises a source of positive pressure.

16. The apparatus according to claim 13 wherein a frit is located between the reaction chamber and the measuring chamber, said frit permitting passage or liquid but not passage of particles;

the method according to claim 1 wherein the particles are incubated with the sample and a linker.

FIG. 1

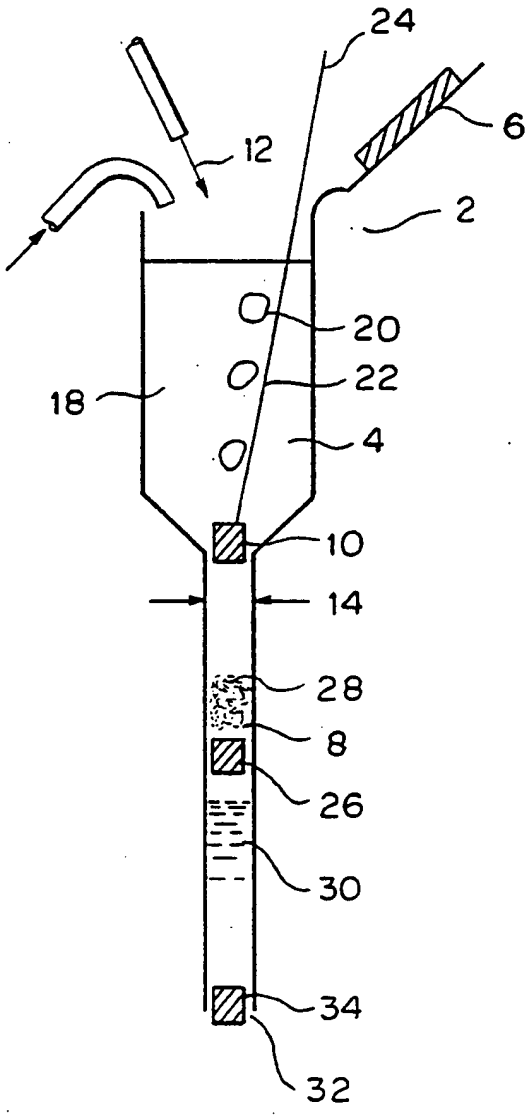


FIG. 2

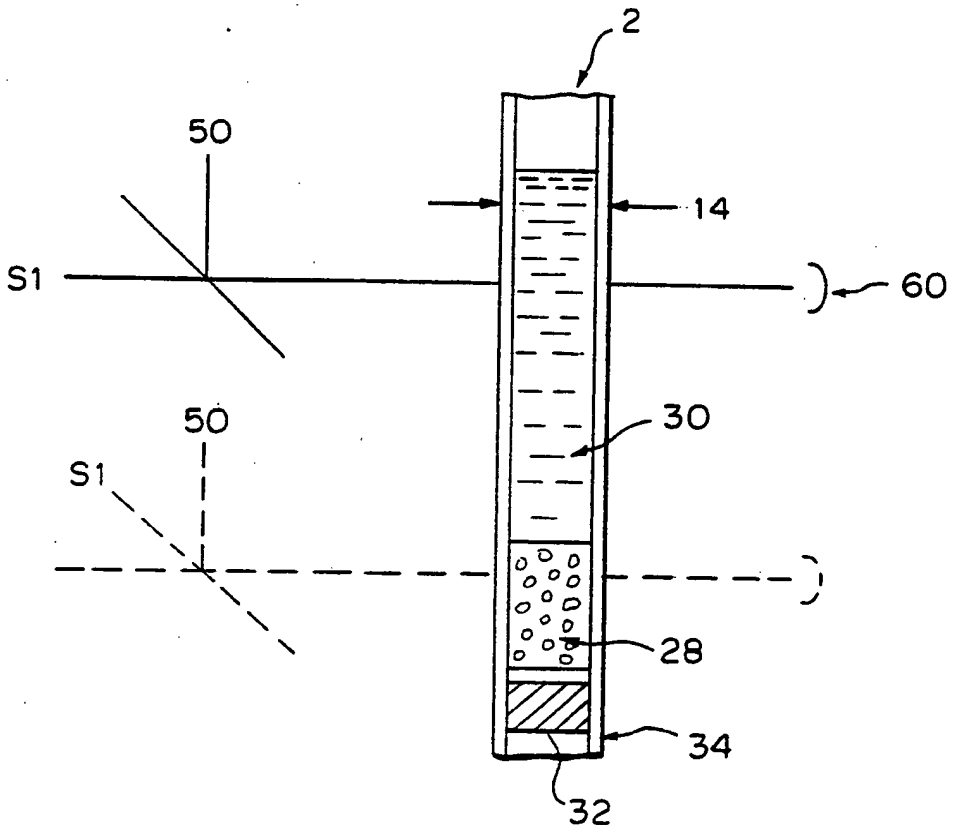
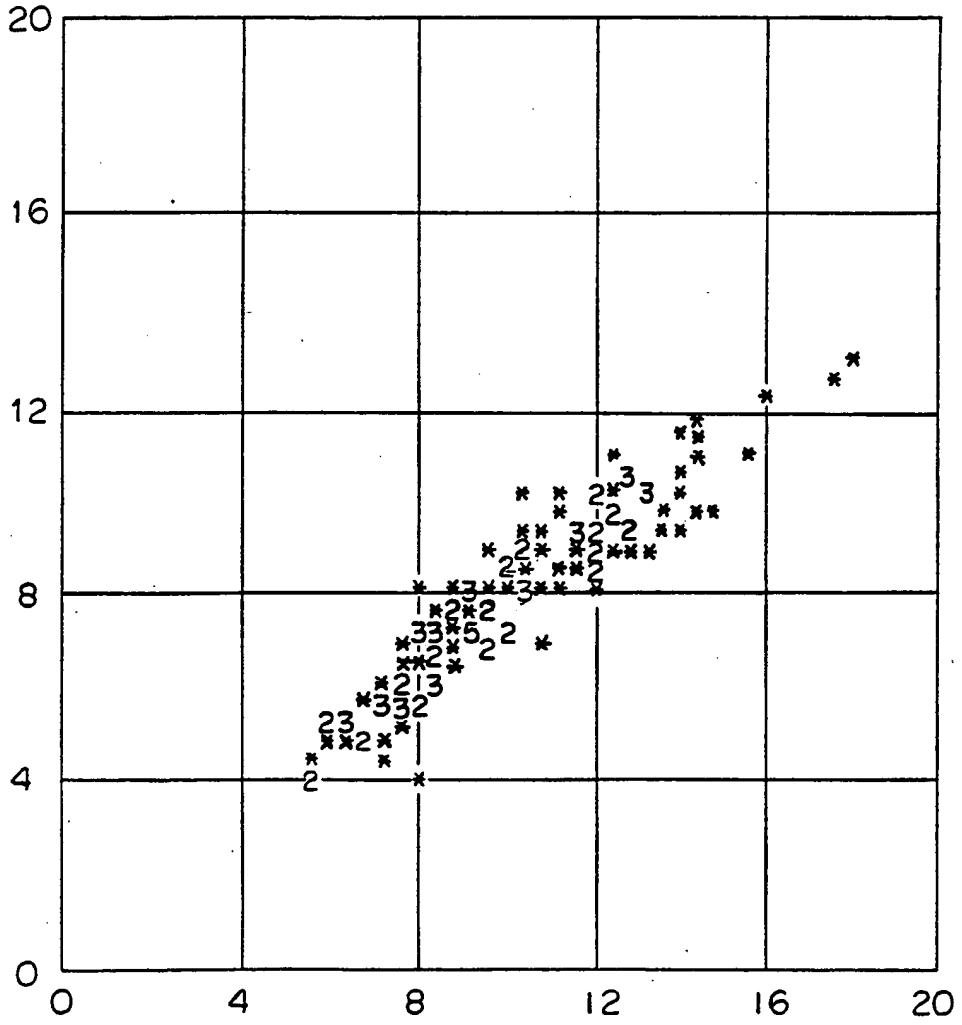
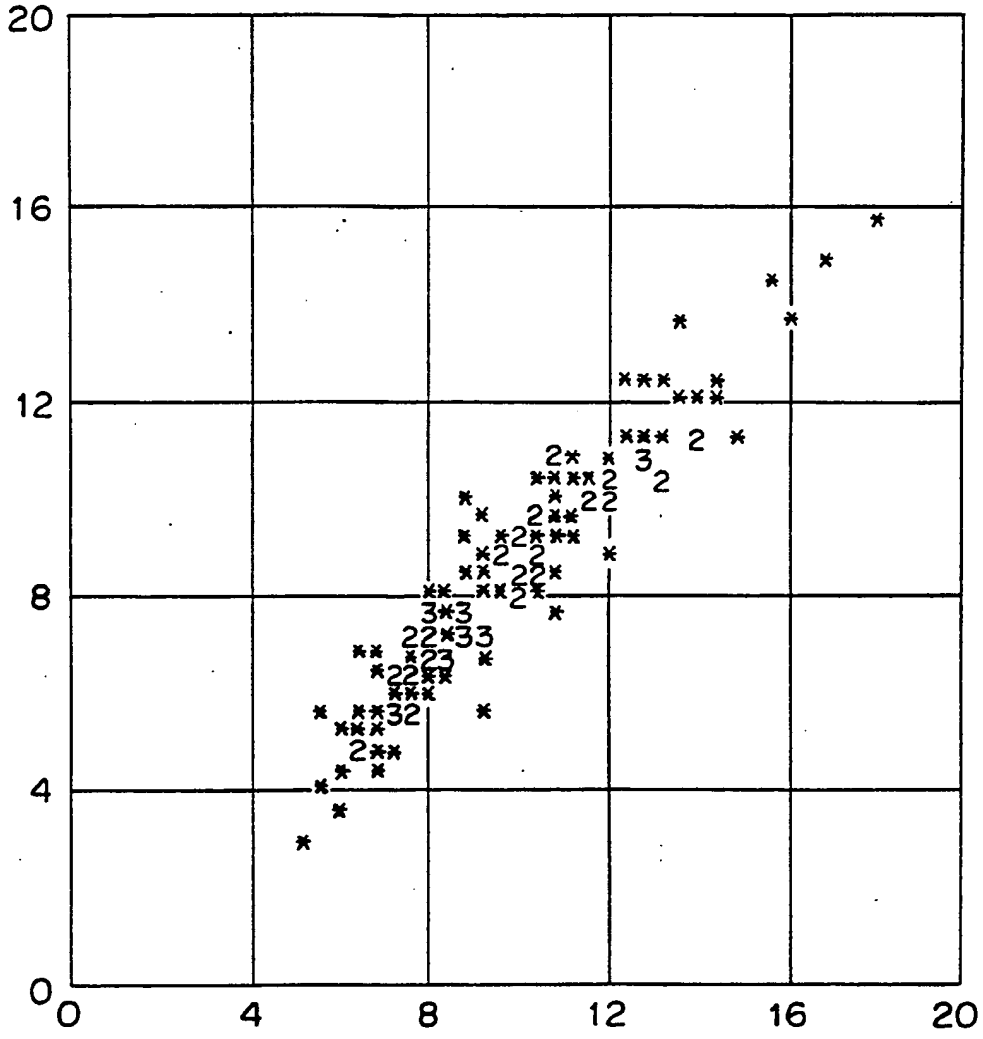


FIG. 3



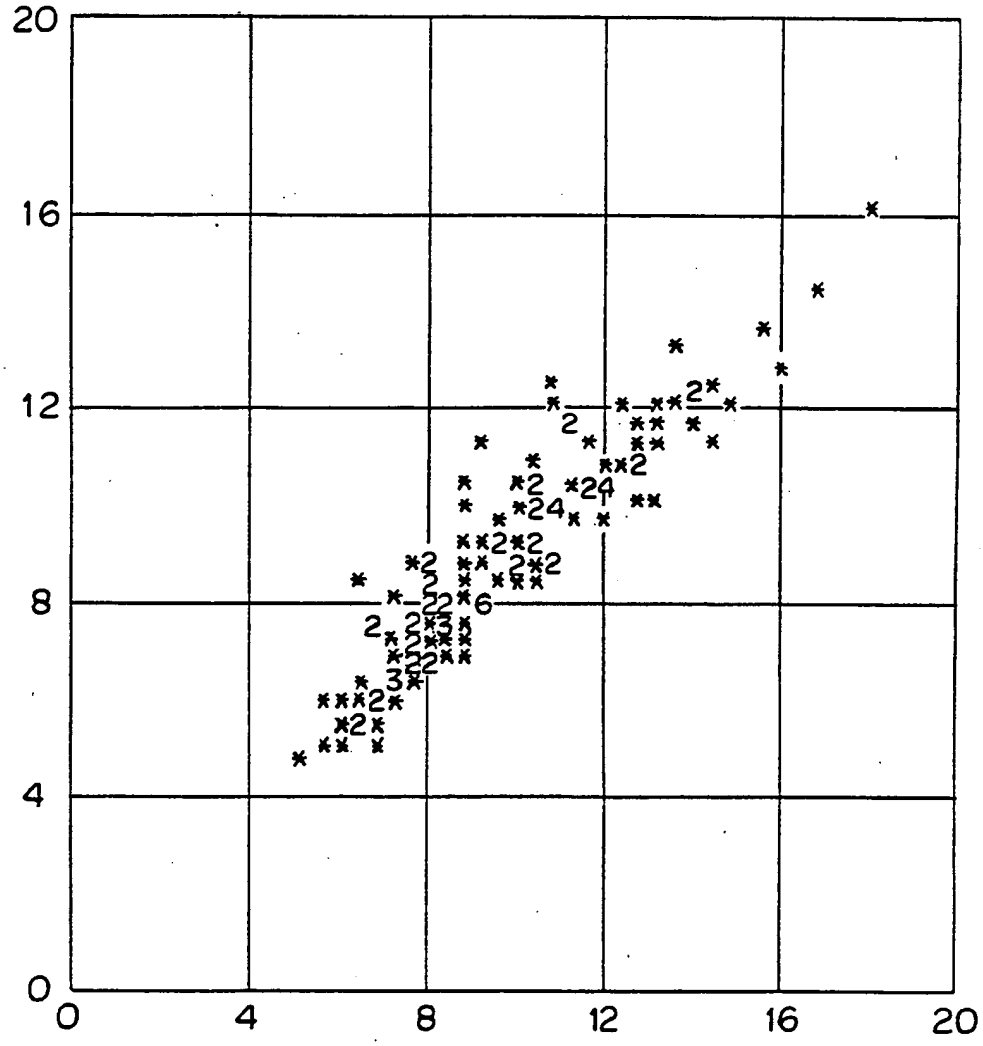
F(X) = .87 + (.69* X)
COEFFICIENT OF DETERMINATION R-SQUARED = .87
COEFFICIENT OF CORRELATION = .93
STANDARD ERROR OF ESTIMATE = .72
NUMBER OF POINTS PLOTTED = 130

FIG. 4



F(X) = -.48 + (.91* X)
COEFFICIENT OF DETERMINATION R-SQUARED = .9
COEFFICIENT OF CORRELATION = .95
STANDARD ERROR OF ESTIMATE = .77
NUMBER OF POINTS PLOTTED = 130

FIG. 5



F(X) = 1.38 + (.78* X)
COEFFICIENT OF DETERMINATION R-SQUARED = .85
COEFFICIENT OF CORRELATION = .92
STANDARD ERROR OF ESTIMATE = .85
NUMBER OF POINTS PLOTTED = 130

FIG. 6

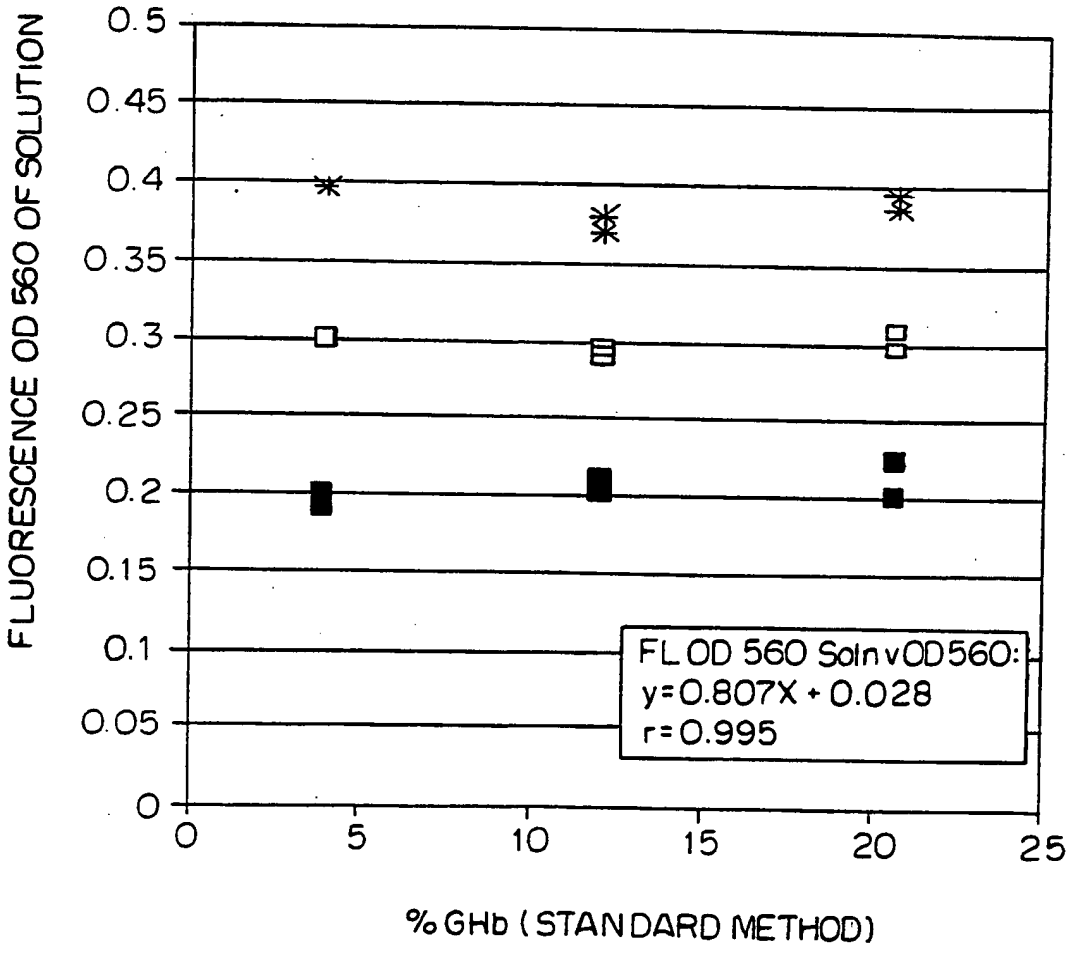

FLUORESCENCE OD 560 OF SOLUTION
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
20
25
FL OD 560 Soln v OD 560:
y = 0.807X + 0.028
r = 0.995
% GHb (STANDARD METHOD)
0.225 OD 560
0.325 OD 560
0.450 OD 560

FIG. 7

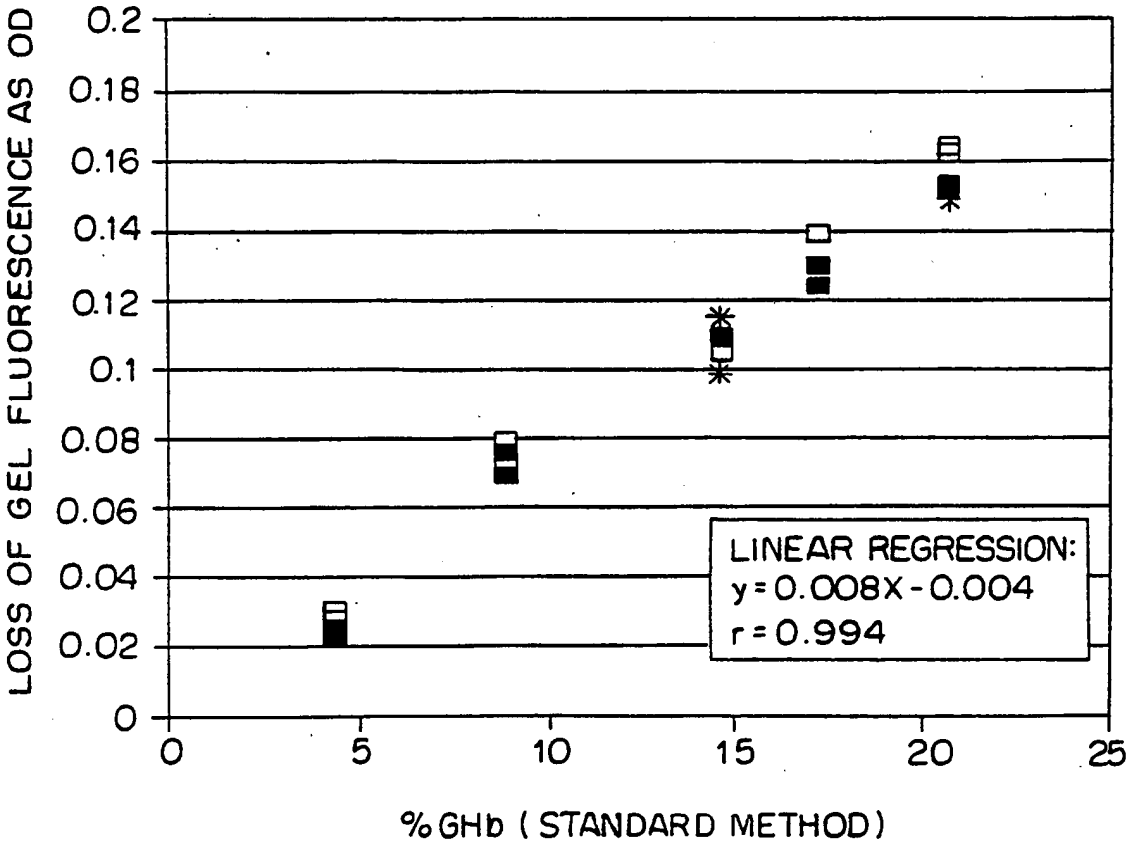

LOSS OF GEL FLUORESCENCE AS OD
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
5
10
15
20
25
%GHb (STANDARD METHOD)
LINEAR REGRESSION:
y = 0.008X - 0.004
r = 0.994
0.225 OD
0.325 OD
0.450 OD

FIG. 8

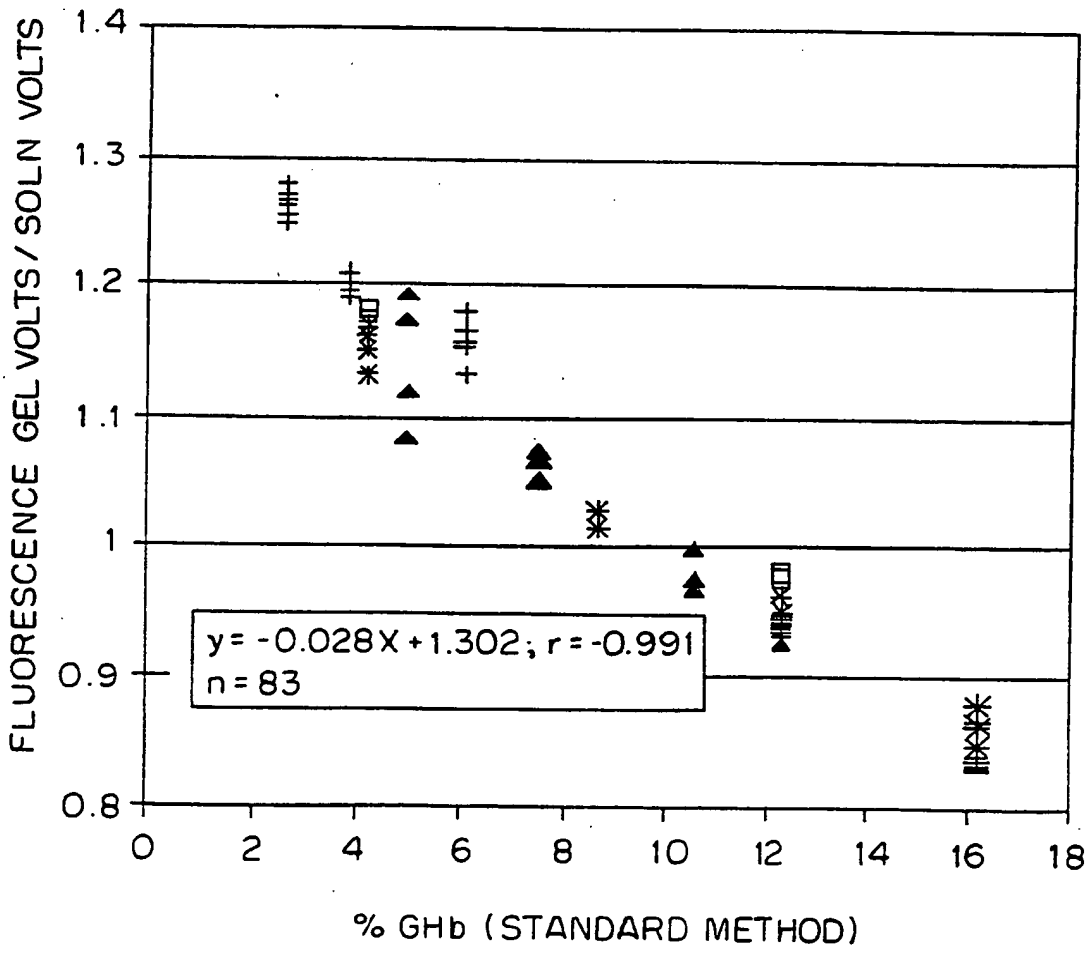
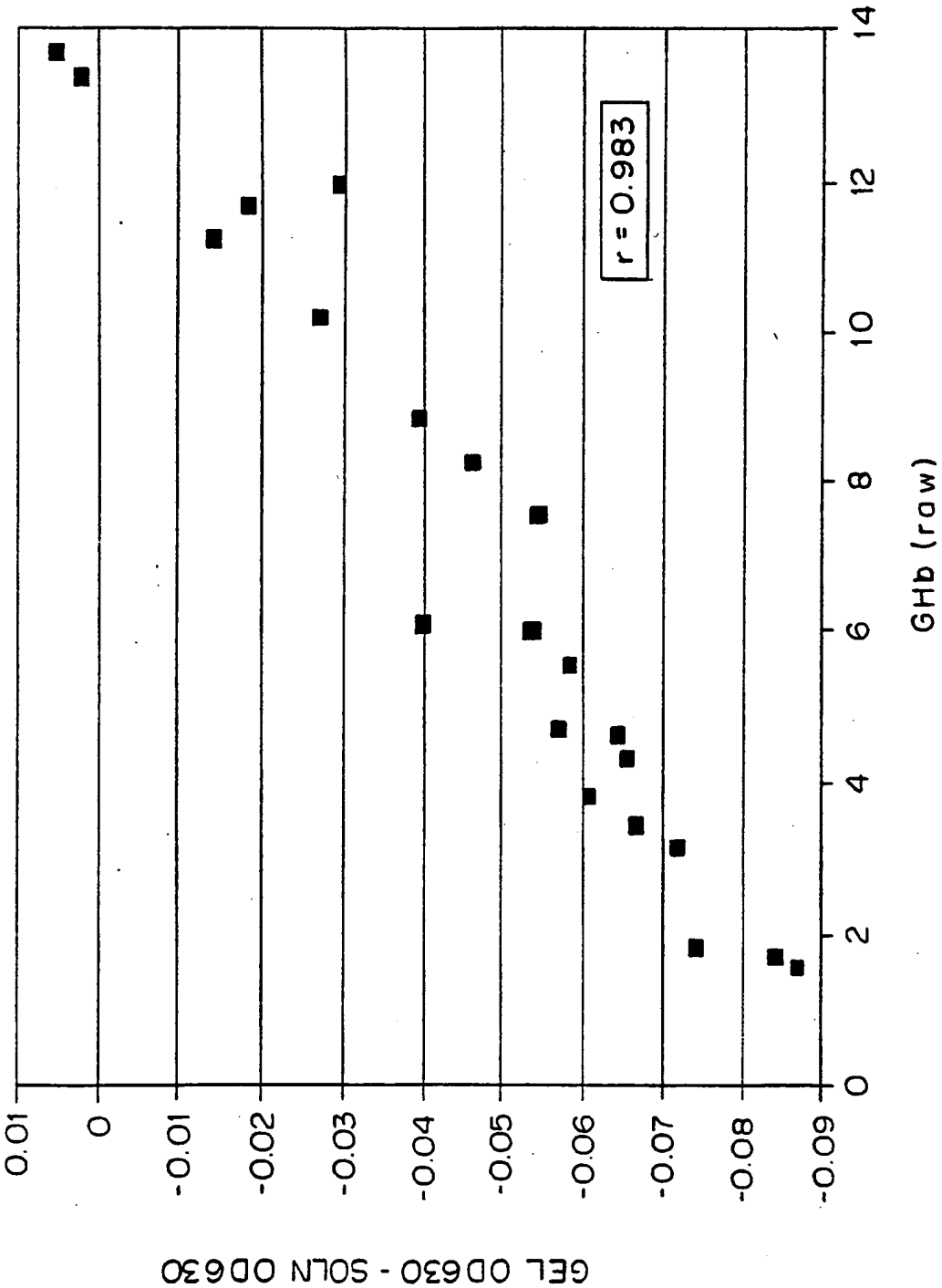

FLUORESCENCE GEL VOLTS / SOLN VOLTS
1.4
1.3
1.2
1.1
1
0.9
0.8
0
2
4
6
8
10
12
14
16
18
y = -0.028X + 1.302; r = -0.991
n = 83
% GHb (STANDARD METHOD)
EXP A
EXP B
EXP C
EXP D

FIG. 9


r = 0.983
GHb (raw)
GEL OD630 - SOLN OD630
0.01
0
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
-0.07
-0.08
-0.09
0
2
4
6
8
10
12
14

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 94/06427

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N33/543 G01N33/58 G01N33/72

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 115, no. 19, 11 November 1991, Columbus, Ohio, US; abstract no. 202730, M. KYUJI 'Method for improving the accuracy of optical measurement in immunoassays.' page 523 ;column 1 ; see abstract & PATENT ABSTRACTS OF JAPAN vol. 15, no. 460 (P-1278) (4988) 21 November 1991 & JP,A,03 195 953 (TOSHIBA CORPORATION) 27 August 1991 see abstract --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 October 1994	07. 11. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Van Bohemen, C

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/06427

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PATENT ABSTRACTS OF JAPAN vol. 9, no. 239 (P-391) (1962) 25 September 1985 & JP,A,60 093 353 (WAKO JUNYAKU KOGYO KK) 25 May 1985 see abstract	1-16
A	EP,A,0 126 450 (I. TRIPATZIS) 28 November 1984 see page 3, line 10 - line 18 see page 8, line 5 - line 11; claims 3,10,22,25	1-16
A	EP,A,0 411 944 (SYNTEX USA INC.) 6 February 1991 see column 1, line 43 - line 45 see column 4, line 39 - line 49 see column 15, line 4 - line 18 see column 24, line 28 - line 41	1-16

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/06427

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0126450	28-11-84	DE-A,C	3322373	22-11-84
		CA-A-	1248873	17-01-89
		DE-A-	3485912	15-10-92
		JP-A-	60035265	23-02-85
EP-A-0411944	06-02-91	JP-A-	3095463	19-04-91